Reference: *Biol. Bull.* **163:** 420–430. (December 1982)

EFFECTS OF ENZYMATIC AND NONENZYMATIC PROTEINS ON *ARBACIA* SPERMATOZOA: REACTIVATION OF AGED SPERM AND THE INDUCTION OF POLYSPERMY*

PHILIP DUNHAM, LEONARD NELSON, LESLIE VOSSHALL, AND GERALD WEISSMANN

Marine Biological Laboratory, Woods Hole, MA 02543

Abstract

Arbacia sperm become inactive after dilution in sea water. We have shown that any of six proteins reactivated the aged sperm as judged by their fertilizing capacity or their motility. In suspensions of inactive sperm in which the mean fertilizing capacity was less than 3%, brief incubation with any of the proteins at 0.5 mg/ml stimulated fertilizing capacity to 70–90%. Reactivation by the proteins was detected at concentrations lower than 2 μg/ml. All six of the proteins also stimulated motility of aged sperm by 30–70%.

The normal block to polyspermy may involve inactivation of sperm by substances released from the eggs during the fertilization reaction. All six proteins tested on inactive sperm were also shown to induce polyspermy in mixtures of eggs and fresh sperm. Whereas in control mixtures with polyspermic cleavage of ~1% of eggs, proteins at 2 mg/ml induced 5–50% polyspermic cleavage, and induction of polyspermy was detected at 5 μg/ml.

The six proteins showing activity included enzymes and also the relatively inert gelatin. The concentration dependence was upon weight/volume and not molarity. Though the mode of action is unclear, it must be rather nonspecific, and is certainly not dependent on enzymatic activity. The same mode of action is likely for activation of aged sperm and induction of polyspermy.

Introduction

Sea urchin sperm suspended in sea water for a few hours become immotile and lose their ability to fertilize eggs (Gemmill, 1900; Gray, 1928a; Rothschild and Tyler, 1954; Bishop, 1962; Branham, 1966; Mann, 1964; Nelson, 1967). Sir James Gray (1928a) entertained the possibility that this "senescence" might be reversible. Although inactivation was ascribed to a "loss of energy reserves" (Gemmill, 1900; Tyler, 1953), it has been suggested that inactive, senescent *Arbacia* sperm can be reactivated by treatments which would not be expected to replenish "energy reserves" (*e.g.* dilution by fresh sea water [Gray, 1928a] or suspension in sea water in which eggs had been incubated [Cohn, 1918; Hathaway, 1963]). However, no satisfactory explanations have been offered which explain the well-documented inactivation, or the less clearly defined reactivation of sperm. Thus, for example, an inhibitory effect of heavy metals has been invoked to explain the inactivation of sperm (Rothschild and Tyler, 1954). While metals certainly may inhibit motility,

Received 3 December 1981; accepted 7 September 1982.

* *We dedicate this paper to the late H. Burr Steinbach, the mentor of two of us (PD & LN).*

Abbreviations: see Table I.

their removal is unlikely to be the basis for the increase in oxygen consumption upon dilution in fresh sea water, the "dilution effect" (Gray, 1928b). Possible clues come from studies in which a wide variety of agents have been shown either to increase the motility of freshly diluted *Arbacia* sperm or slow the onset of inactivation (*e.g.*, Branham, 1966; Steinbach, 1966; Tyler and Tyler, 1966; Nelson, 1972a, 1978; see also Steinbach and Dunham, 1961). Similar observations on motility have been made on sperm from various mammalian and avian species (Schindler and Nevo, 1962; Wales and White, 1962; Liess and Grove, 1963; VanDemark and Koyama, 1963; Garbers *et al.*, 1971; Bavister, 1981).

The normal inhibition of polyspermy in *Arbacia* eggs and those of many other species has long been recognized but remains poorly understood. Two general types of mechanisms might be involved: 1) the surface of the egg, or some portion of it, may be altered subsequent to binding or fusion of one sperm, thereby reducing the probability of penetration of additional sperm; 2) subsequent to contact with one sperm the egg may release an agent or agents which reduce the fertilizing capacity of neighboring sperm. Mechanisms of both types have been proposed. For example, F. R. Lillie described the reversible agglutination of *Arbacia* sperm by a substance released from eggs which he called fertilizin (Lillie, 1913, 1919). Lillie also appreciated that the egg's cortical reaction is too slow to be the only process at the surface of the egg operating to prevent polyspermy (see recent reviews containing discussions of polyspermy by Austin, 1978; Epel, 1978; Schuel, 1978; and Dale and Monroy, 1981).

Doubts have been expressed about the existence of a rapid block to polyspermy in sea urchin eggs (Hagström and Allen, 1956; Dale and Monroy, 1981). A block to polyspermy associated with electrical depolarization, first proposed by Gray in 1922, has recently been demonstrated in *Strongylocentrotus* eggs (Jaffe, 1976); the depolarization and associated block to polyspermy have been suggested to depend on Na (Schuel and Schuel, 1981). However, the validity of these conclusions has been called into question (Dale and Monroy, 1981).

It was shown nearly a century ago, and confirmed many times since, that a wide variety of chemical agents can induce polyspermy in sea urchin eggs (Hertwig and Hertwig, 1887; Just, 1928; Clark, 1936; Rothschild, 1954; Hagström, 1956; Schuel *et al.*, 1976; Coburn *et al.*, 1981). Polyspermy has now been observed in a wide range of animals (mammals as well as invertebrates). The nature of agents with such reactivity is so diverse as to support no single proposed mechanism for polyspermy; rather, the diversity suggests multiple mechanisms by which polyspermy is induced and therefore redundant mechanisms for the normal block to polyspermy. For example, Hertwig and Hertwig (1887) and Hagström and Allen (1956) induced polyspermy with nicotine and Clark (1936) and Hagström (1956) did so with strychnine; Nelson demonstrated stimulation of motility of freshly diluted sperm by nanomolar concentrations of nicotine (Nelson, 1978) and by micromolar concentrations of strychnine (Nelson, 1972a). However, the higher concentrations which may have been necessary for induction of polyspermy (*e.g.* Clark, 1936) inhibit motility (Nelson, 1972a, 1978; Jaffe, 1980).

An agent with very different reactivity, trypsin inhibitor from soy beans, has been shown to induce polyspermy (Hagström, 1956; Vacquier *et al.*, 1972; Schuel *et al.*, 1976). This agent might act by interfering with the cortical reaction (one of the initial events in fertilization), consistent with a role of an esteroprotease in this reaction (Grossman *et al.*, 1973). In another intriguing observation, polspermy was induced by catalase (Coburn *et al.*, 1981), suggesting that the block to polyspermy is due to release of H_2O_2 from the eggs during the fertilization reaction.

We have found that any of several proteins can reactivate inactive sperm. Reactivation was judged from measurements of fertilizing capacity and of motility. The same proteins also induced polyspermy over a similar range of concentrations. The diverse properties of the proteins (from the enzyme catalase to the relatively inert gelatin) suggest that their mode of action is nonspecific. Our results represent the first clear demonstration of reactivation of the fertilizing capacity of inactive sperm. We also provide evidence that induction of polyspermy and reactivation of sperm have a similar basis. However, it is probable that more than one mechanism exists for induction of polyspermy (and therefore that there is more than one mechanism for the physiological block to polyspermy). Finally, the nature of our effective agents requires a reexamination of mechanisms which have previously been proposed for the modulation of the activity of sperm.

Materials and Methods

Gametes. Spermatozoa and eggs were obtained from mature sea urchins (*Arbacia punctulata*) collected by the Department of Marine Resources of the Marine Biological Laboratory.

Sperm: Electrodes from a 12 v A.C. source were placed across the aboral surface of a male sea urchin for 30 sec or less. The sperm released were rinsed into sea water (~15 ml). Numbers of sperm per ml were determined by absorbance of light at 540 nm in a Spectronic 20 Colorimeter (Bausch and Lomb) (Nelson, 1972a).

Eggs: Female sea urchins were inverted over beakers of sea water (50 ml) and injected periviscerally with ~1 ml of 0.5 *M* KCl. The eggs released were washed twice in sea water by suspension and sedimentation at $1 \times g$. Numbers of eggs/ml were calculated from the packed volume of eggs after centrifugation to constant volume with a hand centrifuge and the mean diameter of *Arbacia* eggs (~75 μm; Harvey, 1956).

Inactivation of sperm by aging. Suspensions of sperm diluted in sea water to about 30×10^6 sperm/ml were allowed to stand for one to two days at room temperature (22–25°C).

Fertilizing capacity of sperm. As a measure of the function of sperm, fresh and inactivated, their capacity to fertilize eggs was measured. The method was similar to that of Lillie (1915). These assays were carried out in plastic Petri dishes (35 mm × 10 mm) at room temperature in a total, final volume of 2 ml. Appropriate volumes of sperm suspension (0.05–0.2 ml) were added to give ~10^6 sperm/ml, final density. Agents to be tested for their effect on fertilizing capacity were then added, and the mixtures were incubated at room temperature, usually for 6 minutes. Then eggs were added (0.1–0.2 ml of stock suspension) to a final density of 25,000 eggs/ml. After incubation for 5 minutes, fertilizing capacity of the sperm was assayed by counting the number of eggs (in a field of 100) with a raised fertilization membrane. (In some experiments the eggs were counted again after 90 min for 2-cell stages as a measure of "normal" fertilization.) Bright field illumination in a compound microscope was used at low power. During this study, 49,400 eggs in all were scored (*c.f.* Weissmann, 1981).

We observed that SBTI modified the cortical reaction which occurs upon fertilization, confirming the observations of others (see Epel, 1978, and Schuel, 1978). The lifting of the fertilization membrane was much less pronounced than in control eggs. However fertilization was not prevented by SBTI and subsequent divisions were not modified. None of the other proteins tested modified the cortical reaction.

Motility of sperm. This was determined by a method described earlier (Nelson, 1972b). Aged sperm were first incubated (~6 min) with agents to be tested for their effect on motility. Then the sperm suspension (at 4–8 $\times$ 10^6/ml) was placed in a low centrifugal field (120 $\times$ *g*) at room temperature for 4 minutes. Under these conditions (in which formaldehyde-killed sperm do not sediment), motile sperm tend to move in a centrifugal direction and the immotile sperm remain in the supernatant suspension. Thus the optical density (at 540 nm in a Spectronic 20 Colorimeter) of the supernatant suspension (containing the immotile cells) is inversely related to motility (Nelson, 1972b).

Polyspermy. Polyspermy was assayed in plastic Petri dishes set up as described above for measurement of fertilizing capacity. Sperm were incubated (6 min) with agents to be tested for their promotion of polyspermy. Then eggs were added and the mixtures were incubated for 45–60 minutes. In all cases at least 90% of the eggs were fertilized, and at least 55% (and generally more than 80%) of the eggs cleaved, either reaching the normal two-cell stage, or being readily recognizable as an aberrant form typical of polyspermy (Just, 1928; Clark, 1936). Scoring was made of fields of 100 eggs for: a) unfertilized eggs; b) fertilized eggs, 1-cell stage; c) normally fertilized eggs, 2-cell stage; and d) polyspermic eggs.

Proteins. The proteins tested for their effects on spermatozoan function were added to the assay suspensions from stock solutions made in sea water (up to 10 mg/ml). Table I lists the proteins employed, their approximate molecular weights, and their commercial sources.

Statistical tests. The randomization test for matched pairs (two tailed) was used to determine levels of significance of difference (*P*) from controls caused by treatments with proteins. This is a nonparametric test with 100% power efficiency (Siegel, 1956). Standard errors of means (SEMs), not used in tests for significance of differences, are shown to indicate variability between experiments. The number of separate experiments (on different preparations of cells) is given by "n".

RESULTS

Reactivation by proteins of aged sperm: fertilizing capacity. We confirmed that *Arbacia* sperm diluted in sea water and aged for a number of hours become inactive

TABLE I

Proteins employed in studies on function of Arbacia *spermatozoa, the approximate molecular weights of the proteins, and their commercial sources.*

Protein	Molecular weight	Commercial source	Abbreviation
catalase (prepared from bovine liver)	250,000	Sigma Chemical Co., St.Louis, MO	CAT
crystalline bovine serum albumin	60,000	Sigma	BSA
Cohn fraction V (from bovine serum)	60,000	Sigma	CFV
superoxide dismutase	32,000	Miles Laboratories Ltd., Rep. of S.A.	SOD
Soy bean trypsin inhibitor (type I-S)	21,000	Sigma	SBTI
gelatin (granular)	(indeterminate)	Matheson Coleman & Bell, Norwood, OH	GEL

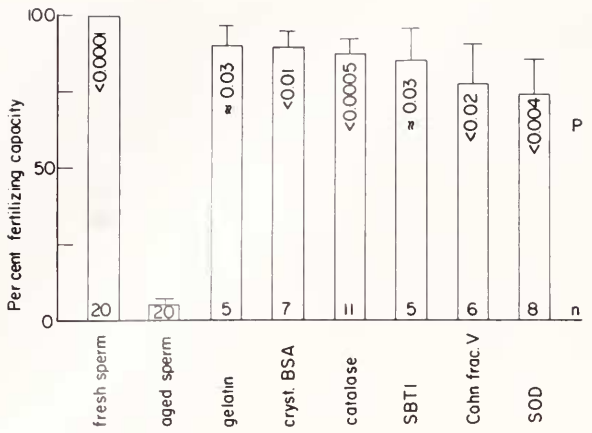

FIGURE 1. Reactivation of the fertilizing capacity of aged *Arbacia* sperm by various proteins, all at 0.5 mg/ml. Procedures for inactivation of sperm by aging and for determination of fertilizing capacity are given in Materials and Methods. Error bars indicate SEMs; n, numbers of experiments. *P* is the level of significance of difference from aged sperm not treated with protein (randomization test for matched pairs).

as judged by their fertilizing capacity. We then discovered that brief incubation of these aged sperm with any of several proteins dramatically restored their fertilizing capacity. Figure 1 shows measurements of fertilizing capacity of fresh sperm, aged sperm (one or two days), and aged sperm incubated 6 minutes with six different proteins (all at 0.5 mg/ml), both enzymatic (CAT and SOD) and nonenzymatic. In preliminary experiments, one other protein, ovalbumin, also reactivated aged sperm. No other proteins were tested.

Preliminary determinations of the time course of reactivation indicated that the full effect was achieved well before 6 minutes. Unfortunately the time required for fertilization by fully active sperm makes an accurate determination of the time course of reactivation impossible.

Figure 2 shows the effect on fertilizing capacity of aged sperm of the proteins in Figure 1 as a function of protein concentration (weight/volume). Reactivating activity was detectable at 5 μg/ml or less. The curves for the various proteins are similar with concentrations expressed as weight/volume despite the wide range of their molecular weights (21,000–250,000; see Table I).

Reactivation of aged sperm: motility. Figure 3 shows measurements of motility of aged sperm reactivated by brief incubation with each of the six proteins used to reactivate fertilizing capacity. The motility of the aged sperm was about 25% of the motility of freshly diluted sperm. All of the proteins increased the motility of aged sperm. Reactivation, judged by motility, is less dramatic in quantity than the reactivation of fertilizing capacity, but it is striking nevertheless.

Despite reactivation of aged, inactive sperm by proteins, we observed, in a preliminary experiment, that aging the sperm in the presence of the proteins did not protect them from eventual inactivation as judged by their motility.

Induction of polyspermy by proteins. Table II shows the results of three typical experiments on induction of polyspermy by three proteins. In addition to the results on polyspermy, Table II shows that the treatment with proteins did not affect fertilization or cleavage. That the variability in per cent polyspermy among these three

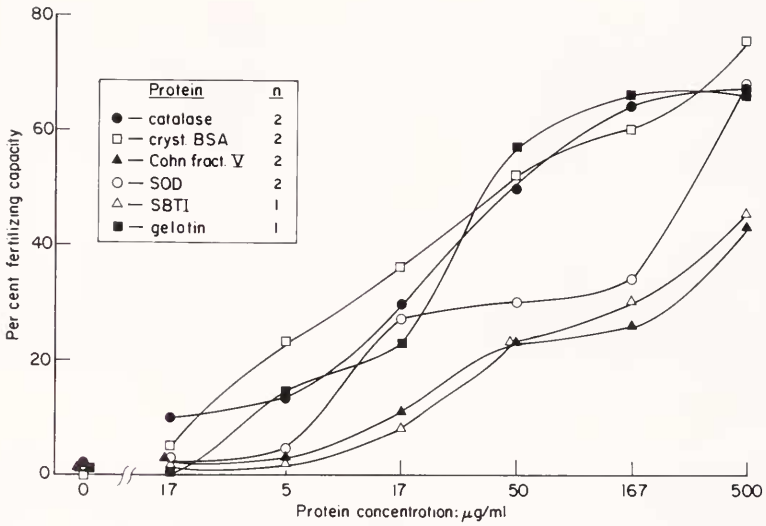

FIGURE 2. Reactivation by proteins of fertilizing capacity of aged *Arbacia* sperm as a function of protein concentration (μg/ml). Procedures for inactivation and for determination of fertilizing capacity are given in Materials and Methods. Protein concentrations on the abscissa are plotted in a logarithmic scale. The inset shows the symbols for the proteins and n, the numbers of experiments for each.

experiments was great is indicated by the standard errors. In these three experiments, however, in no instance was the level of polyspermy induced by a protein in a suspension of sperm and eggs less than 2-fold greater than its control.

Figure 4 shows levels of polyspermy induced by the six proteins (all at 2 mg/ml) in a series of experiments (not every protein was tested in each experiment). Again,

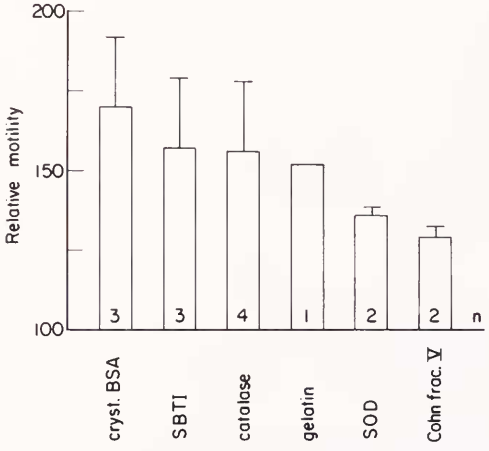

FIGURE 3. Motility of aged *Arbacia* sperm reactivated with proteins, presented relative to the motility of aged sperm, and determined as described in Materials and Methods. The motility of aged sperm not treated with proteins, set at 100, was approximately 25% of the motility of fresh sperm. Numbers of experiments are shown; the error bars show SEMs for BSA, SBTI, and CAT ($n \geq 3$), and total ranges for SOD and CFV ($n = 2$).

TABLE II

Induction of polyspermy by proteins in mixtures of Arbacia *sperm and eggs.*

Condition	Per cent fertilized eggs	Per cent cleavage among fertilized eggs	Per cent polyspermy among cleaved eggs
control	99.0 ± 0.6	93.7 ± 3.8	2.3 ± 1.4
catalase	98.3 ± 1.7	92.3 ± 2.3	22.3 ± 9.4
gelatin	98.0 ± 2.0	93.3 ± 1.8	9.3 ± 2.0
crystalline albumin	99.3 ± 0.3	93.3 ± 2.0	17.1 ± 5.9

Shown are the per cent of eggs fertilized, the per cent of fertilized eggs which had undergone cleavage (either to the normal 2-cell stage or to aberrant polyspermic forms), and the per cent of cleaved eggs which were polyspermic. Results are from suspensions of sperm and eggs incubated with catalase, gelatin, or crystalline albumin (all at 2 mg/ml), and control suspensions. Values are means ± SEMs from 3 experiments.

in no instance was the per cent polyspermy induced by a particular protein less than 2-fold greater than its control (and generally they were much higher). In nine of the thirteen experiments, no polyspermy was observed among 100 eggs in the control suspensions; the highest control level was 4.7%. (The low control levels of polyspermy in Table II and Figure 4 show that there was no problem with overinsemination.) In the four experiments with SOD (the protein least effective at 2 mg/ml in inducing polyspermy), the highest control level was 1.2%, and three were zero; the lowest level with SOD was 2.1%, and its associated control was zero. As shown in Figure 4, there was a high probability of significance of the effects of all the proteins with the possible exception of SOD. In preliminary experiments ovalbumin also induced polyspermy (11.3%; control, 2.2%; n = 3).

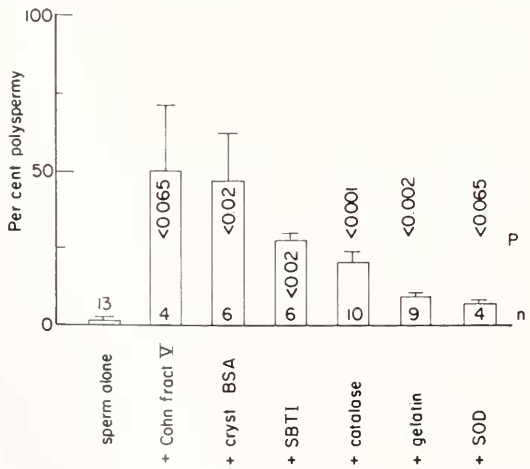

FIGURE 4. Induction of polyspermy in *Arbacia* eggs by various proteins, all at 2 mg/ml. Sperm were freshly diluted and had 100% fertilizing capacity. The experimental design for inducing and quantifying polyspermy is given in Materials and Methods and is illustrated in Table II. The values are means of the per cent polyspermic eggs (aberrant cleavage) of cleaved eggs 45–60 min after mixing sperm and eggs. Error bars represent SEMs; numbers of determinations are also shown. *P* is the level of significance of difference from the control, *i.e.* sperm alone (randomization test for matched pairs).

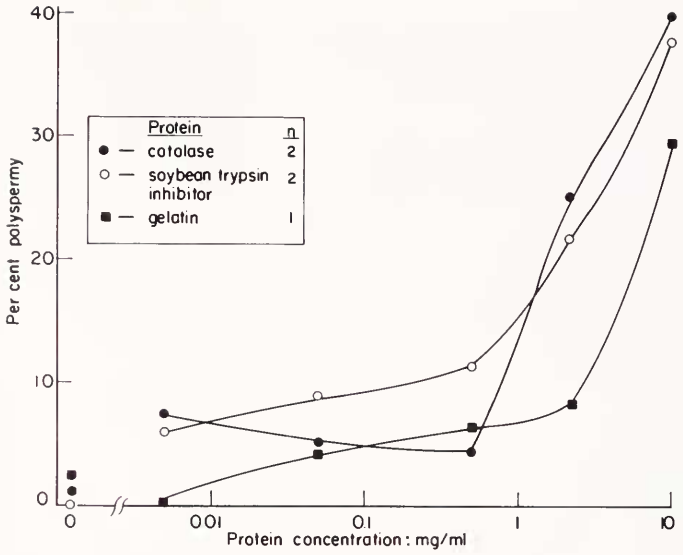

FIGURE 5. Induction by catalase, SBTI, or gelatin of polyspermy in *Arbacia* eggs as a function of the concentrations of the proteins (mg/ml). Sperm were freshly diluted, and had 100% fertilizing capacity. The experimental design is given in Materials and Methods and is illustrated in Table II. The ordinate, "per cent polyspermy," is the per cent of cleaved eggs which were polyspermic (aberrant cleavage). Protein concentrations on the abscissa are plotted in a logarithmic scale. The inset shows the symbols for the proteins and n, the numbers of experiments for each.

Figure 5 shows the dependence of induction of polyspermy on concentration for three of the proteins. The curves describing the dependence on concentration expressed as w/v are similar to one another despite a difference of an order of magnitude in molecular weight between catalase and SBTI (see Table I). The results confirm and extend the recent results from similar experiments (Schuel *et al.*, 1976; Coburn *et al.*, 1981; Schuel and Schuel, 1981), in which catalase and SBTI induced polyspermy. Figure 5 shows that these two proteins induced polyspermy at the lowest concentration tested (5 μg/ml), nearly as low as the concentration (1.7 μg/ml) at which most of the proteins could reactivate the fertilizing capacity of the aged sperm (Fig. 2). The results are in conflict with studies by Coburn *et al.* (1981) and Schuel and Schuel (1981), in which a failure of BSA to induce polyspermy was reported. However, Coburn *et al.*, (1981) reported that boiled catalase (concentration not given) induced <20% polyspermy (and therefore presumably some polyspermy).

DISCUSSION

In this study we show that proteins can promote activity of *Arbacia* sperm. The activities measured were fertilizing capacity, motility, and polyspermy. The proteins stimulated fertilizing capacity and motility of inactive sperm, and induced polyspermic fertilization of eggs by fresh sperm.

Brief incubations (6 minutes or less) with any of six proteins reactivated aged sperm, and no proteins were tested which were ineffective. The diversity of the proteins makes clear the limited specificity of their effect: two are enzymes (CAT, SOD), one is an enzyme inhibitor (SBTI), two are nonenzymatic serum proteins (BSA, CFV), and one (GEL; boiled collagen) is particularly lacking in reactive groups (Miller and Gay, 1982).

The dose-response curves for activation of aged sperm (and for induction of polyspermy, though only 3 proteins were tested) show that the concentration dependence is not on a molar basis, but on concentration as weight/volume. For example, CAT and SOD were about equally effective in reactivating aged sperm at 17 μg/ml (Fig. 2), though their molecular weights differ by nearly an order of magnitude. Similarly CAT and SBTI had comparable activities in inducing polyspermy below 0.1 mg/ml (Fig. 5). Despite this evidence for limited specificity, in most instances the proteins were active in promoting both functions at 5 μg/ml or less. These various considerations make difficult the task of deducing the mechanism of action of the proteins on sperm function. Prevention of binding of inhibitors seems possible but not likely: gelatin is as active in its effects on sperm function as the other proteins, but lacks reactive groups (gelatin is totally lacking in cysteine residues; Miller and Gay, 1982).

Metal chelators can delay inactivation of sperm (Rothschild and Tyler, 1954; Tyler and Tyler, 1966). The relative affinity of such a chelator as ethylene diamine tetracetate (EDTA) for Cu^{++} (the probable inhibitor of sperm in sea water; Rothschild and Tyler, 1954) is 8–10 orders of magnitude higher than for Ca and Mg, the prevalent divalent cations in sea water ($\log_{10} K_{eq}$ of EDTA for Ca: 10.6; for Mg: 8.8; for Cu: 18.7; Martell and Smith, 1974). Therefore, even though the concentrations of Ca and Mg in sea water are ~4 orders of magnitude higher than the concentration of Cu, EDTA would have a much greater effect on [Cu] than on [Ca], and could thereby influence sperm function.

However, it is unlikely that proteins have so pronounced an effect. Most natural amino acids (except cysteine) have about the same affinity for Cu as EDTA has for Mg, and proteins lacking cysteine have a much lower affinity for Cu than do amino acids (for five different pentapeptides the $\log_{10} K_{eq}$ for Cu was about 5.4; Martell and Smith, 1974), and as stated several times above, gelatin, lacking cysteine residues, was as active as the other proteins which contain reactive groups, in its effect on sperm function.

Furthermore, if the mechanism of reactivation of aged sperm by proteins is similar to their mechanism of inducing polyspermy, then chelation of heavy metals cannot be the sole process involved; a role of heavy metals in the block to polyspermy appears unlikely.

Interference with (or binding to) inhibitory substances released from eggs (*e.g.* fertilizin) might explain induction of polyspermy, but cannot explain the activation of aged sperm which have not been in contact with eggs or their products.

Two recent preliminary studies on the effects of bovine serum albumin on rodent sperm suggest a role for proteins in capacitation, *i.e.* preparation for the acrosome reaction (Bavister, 1981; Go and Wolf, 1981). This suggestion may or may not be of relevance to *Arbacia* sperm.

Whatever the mode of action of proteins in inducing polyspermy, our results militate against the suggestions of Coburn *et al.* (1981) and Schuel *et al.* (1976) of specific enzymatic or enzyme inhibitory effects based on induction of polyspermy by CAT and SBTI, since serum albumins and gelatin are also effective (Fig. 5).

Acknowledgments

This work was supported by grants from the NIH (AM 27851 to PD and AM 11949 to GW) and the NSF (PCM 8002358 to LN).

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